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Integrated process for the purification and immobilization of the envelope protein domain III of dengue virus type 2 expressed in *Rachiplusia nu* larvae and its potential application in a diagnostic assay

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ABSTRACT

Dengue incidence has grown dramatically in the last years, with about 40% of the world population at risk of infection. Recently, a vaccine developed by Sanofi Pasteur has been registered, but only in a few countries. Moreover, specific antiviral drugs are not available. Thus, an efficient and accurate diagnosis is important for disease management. To develop a low-cost immunoassay for dengue diagnosis, in the present study we expressed the envelope protein domain III of dengue virus type 2 in *Rachiplusia nu* larvae by infection with a recombinant baculovirus. The antigen was expressed as a fusion to hydrophobin I (DomIIIHFBI) to easily purify it by an aqueous two-phase system (ATPS) and to efficiently immobilize it in immunoassay plates. A high level of recombinant DomIIIHFBI was obtained in *R. nu*, where yields reached 4.5 mg per g of larva. Also, we were able to purify DomIIIHFBI by an ATPS with 2% of Triton X-114, reaching a yield of 73% and purity higher than 80% in a single purification step. The recombinant DomIIIHFBI was efficiently immobilized in hydrophobic surface plates. The immunoassay we developed with the immobilized antigen was able to detect IgG specific for dengue virus type 2 in serum samples and not for other serotypes.

1. Introduction

Dengue is a viral disease transmitted to humans by female mosquitoes mainly of the species *Aedes aegypti* [1]. The incidence of dengue has grown dramatically in the last years and around 390 million infections occur each year [2]. Recently, a vaccine developed by Sanofi Pasteur has become available, but it has been approved only in a few countries (Mexico, The Philippines, Brazil, El Salvador and Costa Rica), while specific antiviral drugs are not available yet [3,4]. Thus, an efficient and accurate diagnosis is very important for the management of the disease and the prevention of epidemics. For dengue diagnosis, serological assays are routinely used because of their simplicity and availability [5,6]. Currently, most of the available serological assays use the whole virus as a source of antigens, which implies high costs associated with virus cultivation and potential biohazard associated with the exposure to infectious viral particles [7]. Moreover, the use of the whole virus decreases diagnosis specificity because of cross reaction with other flaviviruses. On the other hand, the commercially available kits that use recombinant antigens are expensive. Thus, the production of a recombinant antigen in a low-cost platform that avoids whole virus manipulation and enhances specificity becomes an attractive alternative [8,9]. Among the structural proteins of dengue virus (DENV), the domain III (DomIII) of the envelope (E) protein induces serotype-specific antibodies [10] and thus represents an interesting

Abbreviations: *Ac*MNPV, *Autographa californica* multiple nucleopolyhedrovirus; ATPS, aqueous two-phase system; DomIII, envelope protein domain III; dpi, day post-infection; DENV, dengue virus; DENV-2, dengue virus type 2; E, envelope protein of dengue virus; HFBI, hydrophobin I of *Trichoderma reesei*; MOI, multiplicity of infection.

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antigen for the development of a specific diagnostic assay [11–16].

The baculovirus-insect cell system is a versatile eukaryotic expression system for the production of recombinant proteins for biotechnological or pharmaceutical applications [17,18]. Sf9, Sf21 and HiFive insect cell lines are widely used as hosts because of their susceptibility to *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), the most commonly used baculovirus expression vector. However, the main disadvantages of this system at industrial scale are its high cost associated with the use of reactors and the need of tissue-culture facilities [19], and the high risk of contamination. A low-cost alternative is the production of recombinant proteins directly in live insect larvae as "biofactories" [20,21]. Particularly, lepidopteran pest insects, such as *Rachiplusia nu*, which is widely distributed in tropical and subtropical regions of the Americas, are susceptible hosts to *Ac*MNPV infection [22,23]. Previous studies in our lab showed that *R. nu* was an excellent host for the production of horseradish peroxidase, when comparing to three other lepidopteran species (*Spodoptera frugiperda, Helicoverpa zea* and *Heliothis virescens*), since it was susceptible to intrahemocele and oral infection with *Ac*MNPV, reaching high yields in both systems [21,23]. *R. nu* larvae are a destructive plague affecting several economically important crops such as soy and corn. In general, the use of larvae as biofactories yields higher amounts of the recombinant protein than cultured insect cells [24]. However, this technology lacks well-established downstream processes.

Aqueous two-phase systems (ATPS) are an attractive technology that integrates clarification, concentration, and partial purification in one step [25]. Hydrophobins (HFBs) −small amphiphilic proteins of around 7–10 kDa produced by filamentous fungi [26]- can be used as fusion tags for the purification of recombinant proteins by surfactant-based ATPS [27–32]. In these systems, HFB-fused proteins partition to the surfactant-rich phase while the majority of the proteins remain in the aqueous phase. Thus, in a simple purification step, the fusion protein is obtained with high purity and yield. On the other hand, HFBs interact strongly and spontaneously with hydrophobic surfaces, allowing the immobilization of HFB-fused antigens to solid supports commonly used in serological assays (such as enzyme-linked immunosorbent assays-ELISAs) [33,34].

The aim of this work was to produce a recombinant antigen of dengue virus in a low-cost platform for the development of a serological diagnostic assay. Particularly, we assessed the expression of dengue virus type 2 domain III fused to HFBI (DomIIIHFBI) in Sf9 insect cells and *R. nu* larvae. We also studied the purification of DomIIIHFBI by surfactant-based ATPS and showed the application of the recombinant antigen in a specific diagnostic immunoassay.

2. Materials and methods

2.1. Materials

Sf900II insect tissue culture media, the *Spodoptera frugiperda* Sf9 cell line, Cellfectin® and penicillin/streptomycin (ATB/ATM) were from Invitrogen Life Technologies (Gaithersburg, MD, USA). *Rachiplusia nu* larvae were obtained from AgIdea S.A. (Pergamino, Buenos Aires, Argentina). Fetal bovine serum (FBS) was from Internegocios (Buenos Aires, Argentina). Agarplaque Plus and BaculoGold Bright were from BD Biosciences Pharmingen (San Diego, CA, USA). Disposable materials and multiwell plates were from Nunc International (Naperville, IL, USA). Triton X-114 was from Sigma-Aldrich (St. Louis, MO, USA). Dengue Virus Subtype 2 Envelope15 kDa, C-Terminal (Domain III) Recombinant was from Prospec Tany TechnoGene Ltd. (Ness Ziona, Israel). The PageRuler™ Prestained Protein Ladder (10–170 kDa), Cat. number SM0671 used in SDS-PAGEs and Western blots was from Fermentas.

2.2. Expression cassette

The cDNA of DENV-2 E protein (strain 16681, GenBank accession no. U87411.1) was kindly provided by Dr. A. V. Gamarnik (Fundación Instituto Leloir, Argentina). Using this cDNA as a template, the DomIII sequence was amplified by PCR1 with the specific primers 5′CGCGGATCCATGGACAAGCTACAGCTC3' (Primer n°1, sense, BamHI site underlined) and 5′AGAGCCTCCACCTTGGCCGATAGAACTTTCCTT3' (Primer n°2, antisense, HFBI specific sequence underlined). The cDNA of HFBI of *Trichoderma reesei* was kindly provided by Dr. R. Menassa (Agriculture and Agri-Food Canada, Canada), in a plasmid called pjjj161 which contains the gene GFPHFBI [30]. Using this cDNA as a template, the HFBI sequence was amplified by PCR2 with the specific primers 5′TCTATCGGCCAAGGTGGAGGCTCTGGTGGA3' (Primer n°3, sense, DomIII specific sequence underlined) and 5′GGAATTC**CTATCA**CTTCTCAAATTGAGGATG3' (Primer n°4, antisense, EcoRI site underlined, stop codons in bold). The fusion DomIIIHFBI was obtained by overlapping PCR using the products of PCR1 and PCR2 as templates and the specific primers n°1 and n°4. DomIIIHFBI gene was then cloned using BamHI and EcoRI sites into the pAcGP67-B vector (BD Biosciences Pharmingen), which contains a sequence for the glycoprotein 67 (GP67) leader peptide that targets the recombinant protein for secretion (pAcDIIIHFBI).

Another vector containing the expression cassette for green fluorescent protein (GFP) fused to HFBI (GFPHFBI) was constructed to use as a control of the process. The GFPHFBI gene was amplified by PCR using the plasmid pjjj161 as a template and the specific primers 5′CGCGGATCCGTGAGCAACGGCGACGAG (sense, BamHI site underlined) and Primer n°4. GFPHFBI gene was then cloned using BamHI and EcoRI sites into the pAcGP67-B vector (pAcGFPHFBI).

2.3. Virus production

One million Sf9 cells were co-transfected with 2 μg pAcDIIIHFBI and 1 μg linearized BaculoGold Bright DNA (BD Biosciences Pharmingen) in the presence of Cellfectin®. BaculoGold Bright DNA contains the gene for GFP. After a 4-day incubation at 27 °C, the cell culture supernatant was collected and centrifuged at 3000×*g* for 10 min. Co-transfection efficiency was determined by measuring GFP expression by fluorescence under UV light. The recombinant baculovirus polyhedrin-minus vector containing the DomIIIHFBI expression cassette was named AcDIIIHFBI. Following three amplification steps, the virus titer was determined by a plaque assay (1.1×10^8 pfu/ml) [35]. This amplified virus stock was used for the production of the recombinant protein in further experiments.

A recombinant baculovirus vector containing the GFPHFBI expression cassette (AcGFPHFBI) was also produced, following the same protocol and using pAcGFPHFBI vector.

2.4. Insect cell infection

Sf9 suspension cultures (2×10^6 Sf9 cells/ml) grown in Sf900II medium supplemented with 1% of FBS were infected with AcDIIIHFBI at a multiplicity of infection (MOI) of 0.5 or 2 and then incubated in the dark at 27 °C for 6 days or until the day indicated for sample collection. To study the expression among the different day post-infection (dpi), samples of 1 mL were collected each day. The culture supernatant was separated from the cells by centrifugation at 10,000×*g* for 10 min. The pellet and the supernatant were stored at −20 °C until further experiments. For SDS-PAGE and Western blot analysis, the culture supernatant was assessed without any extra treatment while the cell pellet was treated as it is indicated in the next section to obtain total protein extracts. For control purposes, Sf9 suspension cultures infected with AcGFPHFBI